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Bronchoalveolar lavage cell pattern from healthy human lung

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Summary

Bronchoalveolar lavage (BAL) is widely accepted as a key diagnostic procedure in interstitial lung diseases (ILD). We performed a study to obtain reference intervals of differential cell patterns in BAL fluid with special attention to the origin of lavage fluid, e.g. bronchial/alveolar, to atopy and smoking status and to age of the healthy people. We performed bronchoalveolar lavage in 55 healthy subjects with known atopy status (age: 18–64 years, non-smokers/smokers: 34/21) and determined differential cell counts and lymphocyte subsets in BAL fluid and blood. Moreover, in a subgroup of non-smoking healthy individuals we measured the expression of the regulatory T cell marker forkhead box protein 3 (FoxP3) on blood and BAL fluid lymphocytes in addition to a comprehensive set of activation markers. Differential cell counts from the alveolar lavage fraction differed significantly from calculated pooled fractions ($n = 11$). In contrast, marginal differences were found between atopic and non-atopic subjects. Interestingly, the BAL fluid $CD4^+/CD8^+$ ratio correlated strongly with age ($r^2 = 0{\cdot}50$, $P < 0{\cdot}0001$). We consider the bronchial and alveolar fraction to be lavage fluid from fundamentally different compartments and recommend analysis of the alveolar fraction in diagnostic work-up of ILD. In addition, our data suggest that age corrected BAL fluid $CD4^+/CD8^+$ ratios should be used in the clinical evaluation of patients with interstitial lung diseases.

Keywords: activation markers, age, bronchoalveolar lavage, differential cell counts, lymphocyte subsets, reference values

Introduction

Bronchoalveolar lavage (BAL) has become a widely used procedure in various clinical settings, including the differential diagnosis and monitoring of interstitial lung disease (ILD). BAL fluid cell patterns reflect inflammatory cell profiles in affected lung tissues [1] and provide important information that can support the diagnosis of specific ILD [2,3], or exclude other causes of alveolitis. In addition, BAL is used to document specific exposures, such as the identification of asbestos bodies or the proliferative response of BAL fluid lymphocytes to beryllium in chronic beryllium disease (CBD) [4]. Moreover, BAL may be used to investigate inflammatory parameters in infection, neoplasms, exposure to toxic substances, asthma and chronic obstructive pulmonary disease (COPD) [5], and it is useful in monitoring the lung allograft and in evaluating paediatric lung disease [6]. Additionally, BAL can be diagnostic in some rare disorders, e.g. histiocytosis X [7] and pulmonary alveolar proteinosis (PAP) [8].

BAL fluid differential cell counts in ILD usually show variations that differ from patterns found in normal subjects. In 1974, the first paper detailing BAL dealt with normal values, as the authors selected normal subjects and patients undergoing fibreoptic bronchoscopy (FOB) for 'evaluation of intrathoracic lesions' [9]. Clinically healthy controls were included in the study by Van den Bosch and colleagues, performed in our hospital in 1983 [10]. Many groups have been investigating BAL in healthy individuals since then. Issues regarding group size, age, atopy and smoking status have recently been discussed and reviewed by Balbi *et al.* [11]. In addition, there is no consensus on which fractions of BAL fluid should be used for analysis. Furthermore, novel markers for immunophenotyping, such as expression of the integrin CD103 on BAL fluid T cells, have entered the scene and need proper assessment of

reference values in healthy subjects [2,12]. In addition, because activated T lymphocytes play an important role in the pathophysiology of patients with interstitial lung diseases [13–15], we examined the expression of a comprehensive set of activation markers on peripheral blood and BAL fluid T lymphocyte subsets. Finally, we determined forkhead box P3 (FoxP3) expression on BAL fluid T cells. $Fox3^+$-positive regulatory T cells (T_{regs}) play a major role in the control of immune responses against self and exogenous antigens [16]. Deficient T_{regs}, functional or in number, have been reported in different lung diseases [17–19].

In this study we addressed the following issues regarding laboratory parameters and its reference intervals in BAL fluid from healthy human lung: (i) do differential cell counts of pooled lavage fractions differ from cell counts of only the alveolar fraction; and (ii) what demographic variables are of influence on blood and BAL fluid differential cell counts?

Materials and methods

Study population

A group of 55 healthy volunteers with no prior history of pulmonary disease were recruited and checked by an experienced pulmonologist for health status. All subjects were free of diseases based on clinical history, X-ray and lung function tests. None of the volunteers used any pulmonary or anti-inflammatory medication. Bacterial cultures of bronchoalveolar lavage fractions I and II were negative for respiratory pathogens in all subjects. None of the volunteers experienced allergic symptoms of asthma, rhinoconjunctivitis or eczema. Atopy status, i.e. the genetic predisposition to become immunoglobulin (Ig)E-sensitized to allergens commonly occurring in the environment, was assessed by measurements of total serum IgE and allergen-specific IgE (ImmunoCap Phadiatop®, Phadia, Nieuwegein, the Netherlands) levels [20]. The study was approved by the institutional medical ethical committee (registration number: R-06·11A; St Antonius Hospital, Nieuwegein) and all subjects gave written informed consent. Characteristics, pulmonary function and serum laboratory parameters of study subjects are given in Table 1.

Table 1. Characteristics, pulmonary function and serum laboratory parameters of study subjects.

Parameter	Subjects ($n = 55$)
Sex (M/F)	29/26
Age[†]	22 (18–64)
Non-smoker/smoker [pack y]	34/21 [9·6 (2·4)]
Non-atopic/atopic[‡]	45/10
FEV_1 (% pred)	106 (2)
FVC (% pred)	111 (2)
FEV_1/FVC (×100)	95 (1)
Serum IgA (g/l)[†§]	1·97 (1·05–3·93)
Serum IgM (g/l)[†§]	0·96 (0·25–2·14)
Serum IgG (g/l)[†§]	10·5 (6·73–13·7)
Albumin (g/l)[†§]	46 (38–50)
CRP (mg/l)	3

Data are presented as mean (standard error) or as stated otherwise. [†]Data are presented as median (range). [‡]Atopy status was determined by measuring total serum immunoglobulin (Ig)E ≥200 kU/l and/or allergen-specific IgE (Phadiatop®) ≥2·5). [§]Data were not significantly different from established reference intervals. CRP, C-reactive protein; FEV_1, forced expiratory volume in 1 s as percentage of normal predicted value; FVC, forced vital capacity as percentage of normal predicted value; FEV_1/FVC, ratio of FEV_1 to FVC.

Bronchoalveolar lavage

All subjects underwent bronchoscopy and BAL procedure with a flexible bronchoscope according to internationally accepted guidelines [21]. The procedure involved premedication (20 mg codeine per os), and local anaesthesia of the larynx and lower airways (0·5% tetracaine in the oropharynx, 8 cc 0·5% tetracaine in lower airways). Transcutaneous oxygen saturation was monitored continuously by oximeter with a finger probe. BAL was performed in the right middle lobe with a total volume of 200 ml of sterile isotonic saline solution (37°C). BAL fraction I, returned after instilling 50 ml of saline, and BAL fraction II, returned after instilling 3 × 50 ml of saline, were collected in a siliconized specimen trap and immediately kept on ice. An aliquot of both fractions was used for bacterial culture. BAL fluid fractions were filtered through nylon gauze and centrifuged (10 min at 400 ***g*** at 4°C). The cell pellet was washed twice, counted and resuspended in minimal essential medium/RPMI-1640 (Gibco, Grand Island, NY, USA), supplemented with 0·5% bovine serum albumin (Organon, Teknika, Boxtel, the Netherlands). Cells were counted in a Bürker chamber. Cell yield was determined by total cell number per fraction/total recovered volume per fraction. Cell viability was determined by Trypan blue exclusion. Smears for cell differentiation were prepared by cytocentrifugation (Shandon, Runcorn, UK). Cell differentiation was performed by microscopy on cytospinslide after staining with May–Grünwald–Giemsa (Merck, Darmstadt, Germany); at least 2 × 500 cells were counted (Fig. 1). In all

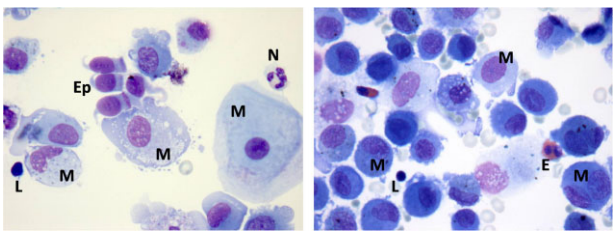

Fig. 1. Representative pictures of cell composition of bronchoalveolar lavage (BAL) fraction I (left) and II (right). L: lymphocyte; M: macrophage; E: eosinophil; Ep: epithelial cell; N: neutrophil.

subjects bronchoscopy and BAL was well tolerated. The cell counts and established reference intervals in Table 2 are based on BAL fluid fraction II.

Peripheral blood

Before the BAL procedure, peripheral blood was drawn for routine analysis of leucocyte cell differentiation (LH750 analyser, Beckman Coulter, Mijdrecht, the Netherlands) and serum analytes [IgA, IgM, IgG (Immage 800, Beckman Coulter), albumin and C-reactive protein (CRP) (Cobas C 501, Roche Diagnostics, Almere, the Netherlands)].

Flow cytometry

To determine lymphocyte subsets in peripheral blood and BAL cellular fraction, cells were labelled with monoclonal fluorochrome conjugated antibodies directed against CD3, CD4, CD8, CD19, CD45, CD(16+56) and CD103 [Becton Dickinson (BD) Biosciences, Alphen aan den Rijn, the

Table 2. Differential cell counts and lymphocyte subpopulations in blood and bronchoalveolar lavage (BAL) fluid in adults (18–64 years).

Non-smokers	Peripheral blood†		Bronchoalveolar lavage		
$n = 34$	#‡	%	%	#§	
			58 (46–69)		Recovery
Leucocytes	5·6 (3·9–7·3)			9·8 (4·7–18·0)	Cells/ml
			95 (90–98)		Vitality
Monocytes	0·4 (0·3–0·8)	7·8 (6·2–12·8)	85·4 (65·3–95·4)	7·5 (4·1–15·9)	Macrophages
Lymphocytes	1·7 (1·3–2·4)	33·9 (23·3–41·5)	11·7 (3·0–32·4)	0·9 (0·3–3·7)	Lymphocytes
Neutrophils	3·0 (2·0–4·7)	54·6 (46·6–62·9)	1·3 (0·2–4·3)	0·2 (0–0·4)	Neutrophils
Eosinophils	0·14 (0·05–0·29)	2·6 (1·1–4·8)	0·3 (0·1–3·5)	0·03 (0·01–0·33)	Eosinophils
Basophils	0·03 (0·02–0·08)	0·6 (0·3–1·8)	0 (0–0·2)	0 (0–0·03)	Basophils
			0	0	Plasma cells
$CD3^+$	1·3 (0·9–2·0)	76 (69–85)	95 (90–98)	0·9 (0·3–3·5)	$CD3^+$
$CD4^+$	0·8 (0·6–1·4)	47 (37–61)	54 (35–79)	0·5 (0·2–1·7)	$CD4^+$
$CD8^+$	0·4 (0·2–0·6)	24 (15–35)	36 (15–57)	0·3 (0·04–1·7)	$CD8^+$
$CD4^+/CD8^+$		1·9 (1·2–3·8)	1·5 (0·6–5·5)		$CD4^+/CD8^+$
$CD19^+$	0·2 (0·1–0·4)	12 (8–20)	2 (0·5–3)	0·02 (0·004–0·09)	$CD19^+$
$CD3^-CD16^+CD56^+$	0·2 (0·1–0·4)	10 (4–17)	5 (2–8)	0·04 (0·02–0·15)	$CD3^-CD16^+CD56^+$
			36 (24–55)	0·35 (0·08–1·77)	$CD103^+$
			0·15 (0·05–0·27)		$CD103^+CD4^+/CD4^+$
			0·7 (0·3–3·0)		BAL/PB $CD4^+/CD8^+$
Smokers	Peripheral blood†		Bronchoalveolar lavage		
$n = 21$	#‡	%	%	#§	
			55 (37–65)		Recovery
Leucocytes	7·1 (4·7–9·0)			19·7 (8·1–45·5)	Cells/ml
			95 (87–98)		Vitality
Monocytes	0·5 (0·5–0·8)	8·3 (6·2–10·1)	96·1 (79·0–98·4)	19·0 (7·3–44·7)	Macrophages
Lymphocytes	2·0 (1·5–2·8)	30·9 (20·3–45·9)	2·3 (0·7–16·5)	0·5 (0·2–1·7)	Lymphocytes
Neutrophils	4·1 (2·0–5·8)	60·5 (39·2–69·2)	0·9 (0·4–3·1)	0·2 (0–0·7)	Neutrophils
Eosinophils	0·16 (0·08–0·32)	2·3 (1·2–5·3)	0·2 (0–1·3)	0·05 (0–0·25)	Eosinophils
Basophils	0·03 (0·02–0·07)	0·4 (0·3–0·9)	0 (0–0·5)	0 (0–0·07)	Basophils
			0	0	Plasma cells
$CD3^+$	1·5 (1·2–2·1)	77 (66–83)	96 (87–99)	0·6 (0·3–1·5)	$CD3^+$
$CD4^+$	1·0 (0·6–1·4)	46 (39–61)	44 (25–58)	0·2 (0·1–0·6)	$CD4^+$
$CD8^+$	0·5 (0·3–0·8)	23 (17–32)	46 (34–70)	0·2 (0·1–0·9)	$CD8^+$
$CD4^+/CD8^+$		1·9 (1·4–3·4)	0·9 (0·4–1·5)		$CD4^+/CD8^+$
$CD19^+$	0·3 (0·1–0·4)	13 (7–19)	1 (0·1–2)	0·005 (0–0·05)	$CD19^+$
$CD3^-CD16^+CD56^+$	0·2 (0·1–0·4)	8 (4–14)	5 (1–12)	0·02 (0·01–0·11)	$CD3^-CD16^+CD56^+$
			41 (30–68)	0·19 (0·04–0·70)	$CD103^+$
			0·19 (0·07–0·41)		$CD103^+CD4^+/CD4^+$
			0·4 (0·1–1·3)		BAL/PB $CD4^+/CD8^+$

Differential cell counts and lymphocyte subpopulations, and established reference intervals in BAL fluid fraction II. Values are expressed as median (10th–90th percentiles). †Reference intervals in peripheral blood did not differ significantly from the established reference intervals used in our hospital [27]; ‡$\times 10^6$ cells/ml. §$\times 10^4$ cells/ml.

Netherlands]. In a subgroup of non-smoking healthy individuals, additional staining was performed with antibodies directed against CD25, CD28, CD69, very late antigen-1 (VLA-1) and FoxP3 (BD Biosciences). Flow cytometry was performed according to standard protocols and within 24 h of fixation to detect and quantify labelled cells [fluorescence activated cell sorter (FACS)Calibur, Becton Dickinson]. At least 100 000 events were analysed with FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Statistical analysis

The majority of differential cell counts/lymphocyte subset values were not normally distributed. The cell counts and established reference intervals are expressed as median (10th–90th percentiles). The Mann–Whitney *U*-test was used to compare peripheral blood (PB) and BAL fluid cell populations. Correlations between different variables were determined using Spearman's rank coefficient. We compared the distribution of cell populations in fractions I and II as well as fraction II with calculated pooled fractions (I + II) using the paired *t*-test. The absolute cell counts and the percentages of different cell populations in the pooled fractions were calculated according to the following formulae:

Absolute cell population numbers (10^4/ml):

$$\frac{\left[\text{cell population fraction I}\,(\%)\times\text{cell yield fraction I}\,(10^6\ \text{cells})+\ \text{cell population fraction II}\,(\%)\times\text{cell yield fraction II}\,(10^6\ \text{cells})\right]}{\left[\text{recovery fraction I}\,(\text{ml})+\text{recovery fraction II}\,(\text{ml})\right]}$$

Proportion of cell population (%):

$$\frac{\left[\text{cell population fraction I}\,(\%)\times\text{cell yield fraction I}\,(10^6\ \text{cells})+\ \text{cell population fraction II}\,(\%)\times\text{cell yield fraction II}\,(10^6\ \text{cells})\right]}{\left[\text{cell yield fraction I}\,(10^6\ \text{cells})+\text{cell yield fraction II}\,(10^6\ \text{cells})\right]}$$

The statistical evaluation of our data was performed using PASW statistics 17·0 (SPSS, Inc., Chicago, IL, USA) and Graphpad Prism version 5 (Graphpad Software, Inc., San Diego, CA, USA) software packages.

Results

Differential cell counts and lymphocyte subpopulations in BAL fluid and in blood

Smoking significantly affects BAL fluid cell profiles in different lung diseases [22–26]. Here we provide separate reference intervals of BAL fluid differential cell counts and lymphocyte subpopulations for non-smokers and smokers (Table 2). Reference values for the BAL fluid $CD103^+CD4^+$/$CD4^+$ ratio and the BAL/PB $CD4^+/CD8^+$ ratio, a potential new diagnostic marker in interstitial lung diseases [2,12], are summarized in Table 2. Peripheral blood differential cell counts and lymphocyte subpopulations did not differ from reference intervals used in our hospital [27].

Differential cell counts in fraction I and fraction II

Some investigators have considered the first aliquot as representing predominantly bronchial airway cells and secretions and have kept this aliquot separate, whereas subsequent sequential aliquots (usually three to four in number) are considered more representative of distal airspaces ('alveolar sampling') and pooled for subsequent cellular analysis [28,29]. Other centres pool all retrieved BAL aliquots prior to submitting BAL for laboratory analysis. Table 3 illustrates a different cell distribution between fraction I and fraction II, indicating sampling of different airway compartments.

Figure 2 illustrates BAL fluid cell counts if fractions I and II would have been pooled compared with the corresponding cell counts of only fraction II in 11 healthy non-smokers. Pooling resulted in lower absolute macrophage and lymphocyte counts ($P = 0{\cdot}008$ and $P = 0{\cdot}04$, respectively). Borderline significance was observed for the percentage of lymphocytes ($P = 0{\cdot}05$). Neutrophil and eosinophil cell counts revealed no differences comparing the pooled fractions and fraction II.

Therefore, in healthy subjects, fractions I and II have a different cell distribution and dilution of fraction II with fraction I has a substantial effect on cell counts. Together, these results indicate that fractions I and II should not be pooled.

Correlation of demographic variables with blood and BAL fluid composition

Age. The influence of age on BAL cell composition has been a subject of considerable debate [30]. Analysis of correlations between age and differential cell counts or lymphocyte subsets showed strong positive and negative correlations between age and, respectively, the percentage of $CD4^+$ and $CD8^+$ BAL fluid lymphocytes and subsequently the BAL fluid $CD4^+/CD8^+$ ratio in healthy non-smokers ($r^2 = 0{\cdot}50$, $P < 0{\cdot}0001$) (Fig. 3). This effect could be ascribed to lower absolute BAL fluid $CD8^+$ cells [>40 years *versus* <30 years, median (range); 0·2 (0·02–1·1) *versus* 0·5 (0·1–2·7), $P < 0{\cdot}0001$] along with similar BAL fluid $CD4^+$ cell numbers [0·4 (0·1–3·7) *versus* 0·5 (0·1–3·3)]. A resembling pattern was observed in lymphocyte subset counts in peripheral blood comparing the two age groups: lower absolute $CD8^+$ cells and similar absolute $CD4^+$ cell numbers [0·3 (0·2–0·6) *versus* 0·5 (0·3–0·9), $P = 0{\cdot}01$ and 0·8 (0·5–1·6) *versus* 0·8 (0·5–1·4), respectively]. Moreover, the $CD103^+CD4^+/CD4^+$ ratio (Fig. 3) correlated positively with age and the recovery of the alveolar fraction correlated negatively with age ($r^2 = 0{\cdot}21$, $P = 0{\cdot}006$). We found no confounding effect of the recovery of the alveolar BAL fraction on the described parameters.

Table 3. Bronchoalveolar lavage (BAL) fluid cell percentages of fraction I *versus* fraction II in adults (18–64 years).

Non-smokers	Fraction I	Fraction II	
$n = 34$	%	%	*P*-value*
Macrophages	85·0 (61·2–95·3)	85·4 (65·3–95·4)	n.s.
Lymphocytes	5·2 (1·1–21·6)	11·7 (3·0–32·4)	<0·0001
Neutrophils	4·9 (1·1–25·9)	1·3 (0·2–4·3)	0·001
Eosinophils	0·2 (0–7·6)	0·3 (0·1–3·5)	n.s.
Basophils	0 (0–0·3)	0 (0–0·2)	n.s.
Plasma cells	0	0	n.s.
Smokers	Fraction I	Fraction II	
$n = 21$	%	%	*P*-value*
Macrophages	91·7 (75·5–98·0)	96·1 (79·0–98·4)	n.s.
Lymphocytes	1·9 (0·2–8·5)	2·3 (0·7–16·5)	n.s.
Neutrophils	4·8 (0·8–15·7)	0·9 (0·4–3·1)	0·001
Eosinophils	0·4 (0–3·3)	0·2 (0–1·3)	n.s.
Basophils	0·1 (0–0·6)	0 (0–0·5)	n.s.
Plasma cells	0	0	n.s.

*Difference between fraction I and II. Data are presented as median (10th–90th percentiles). n.s., not significant.

None of the other parameters correlated strongly with age (data not shown).

Atopy. In western countries, atopy seems increasingly present in the general population and its presence is relevant for all the biological methodologies [11]. Marginal differences were found between subjects with increased and subjects with normal IgE/phadiatope values comparing differential cell counts or lymphocyte subsets. In BAL fluid, only the percentage of neutrophils was significantly higher in atopic individuals who smoked ($P = 0{\cdot}01$). In peripheral blood, IgA and albumin levels were higher in atopic smokers ($P = 0{\cdot}04$ and $P = 0{\cdot}03$), but still within the established reference intervals. The percentage of circulating monocytes and basophils and the absolute monocyte counts were higher in atopic non-smokers ($P = 0{\cdot}02$, $P = 0{\cdot}02$, and $P = 0{\cdot}01$, respectively).

Smoking status. Smokers had increased absolute cell numbers in BAL fluid ($P < 0{\cdot}0001$). In addition, the percentage of macrophages was higher, resulting in a threefold higher number of macrophages in smokers compared to non-smokers. Furthermore, smokers had lower percentages of lymphocytes ($P < 0{\cdot}0001$), $CD19^+$ cells ($P = 0{\cdot}0004$) and $CD4^+$ cells ($P = 0{\cdot}006$) and a higher percentage of $CD8^+$ cells ($P = 0{\cdot}005$). Consequently, the $CD4^+/CD8^+$ ratio in BAL fluid of smokers was lower compared to non-smokers ($P = 0{\cdot}005$).

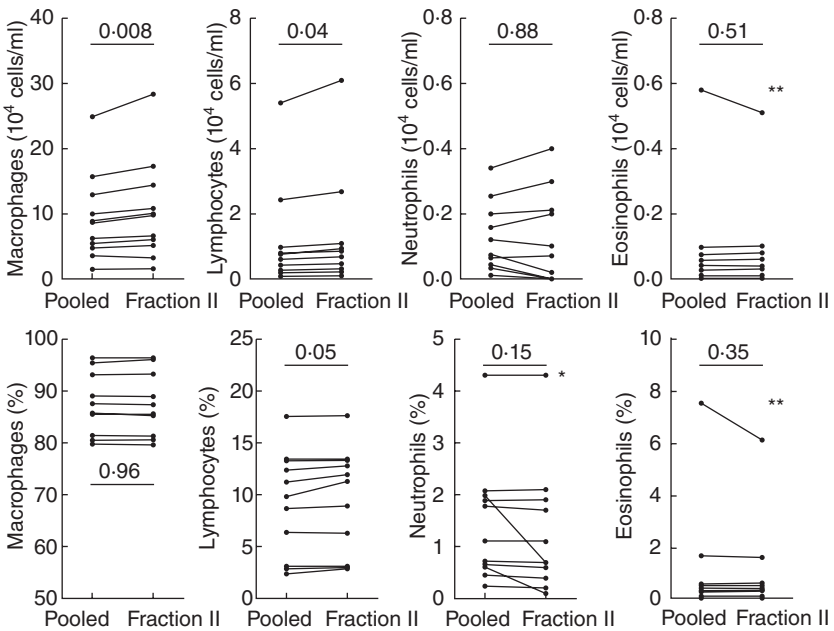

Fig. 2. Bronchoalveolar lavage (BAL) fluid differential cells counts from pooled fractions (calculated) *versus* corresponding fractions II in healthy non-smokers ($n = 11$). The relatively high neutrophil percentages (*) and eosinophil counts (**) are from atopic individuals.

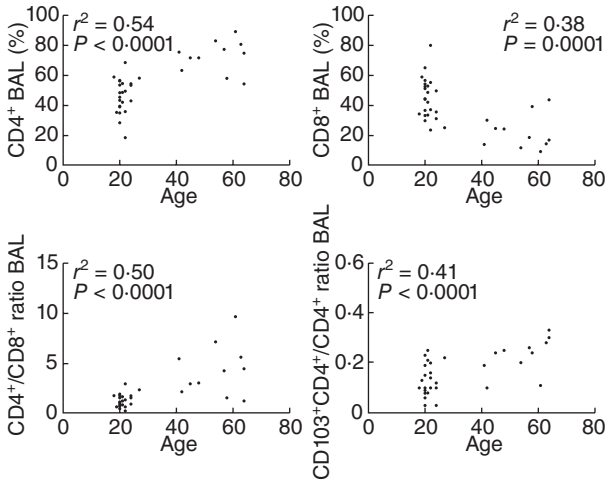

Fig. 3. Scatterplots illustrating strong correlations between age and the percentage of bronchoalveolar lavage (BAL) fluid $CD4^+$ lymphocytes, $CD8^+$ lymphocytes, the $CD4^+/CD8^+$ ratio and the $CD103^+CD4^+/CD4^+$ ratio in healthy non-smokers.

In peripheral blood, the main difference observed between smokers and non-smokers was a higher absolute leucocyte count ($P = 0{\cdot}0002$) and a higher percentage and number of neutrophils (respectively, $P = 0{\cdot}03$ and $P = 0{\cdot}0004$).

Gender. No significant differences were observed between males and females of any parameter measured (data not shown).

Expression of activation markers and FoxP3 on blood and BAL fluid lymphocytes

Expression of activation markers on lymphocytes. Table 4 presents the percentage of peripheral blood and BAL fluid $CD4^+$ and $CD8^+$ T lymphocytes that expressed activation markers. CD69 and VLA-1 expression was higher on $CD4^+$ and $CD8^+$ BAL fluid cells compared to corresponding PB lymphocytes. Moreover, $CD8^+CD25^+$ cells were increased in BAL fluid compared to blood. Finally, CD28 expression was decreased on BAL fluid lymphocyte subsets *versus* PB lymphocyte subsets.

Naturally occurring regulatory T cells in blood and BAL fluid. Both the median fluorescence intensity of FoxP3 on $CD4^+$ cells and the percentage of $CD4^+FoxP3^+$ cells were higher in BAL fluid samples *versus* PB samples (Fig. 4 and Table 4).

Discussion

We established reference intervals of commonly or increasingly used cellular inflammation markers in BAL fluid in a group of healthy adults. Strong correlations were found between age and BAL fluid lymphocyte subsets. This may suggest the use of age corrected $CD4^+/CD8^+$ ratios in the clinical evaluation of patients with interstitial lung diseases. Finally, we showed that pooling of bronchial and alveolar fractions significantly alters some cell differential counts compared to cell counts of the sole alveolar fraction.

In 1983, healthy volunteers were included in the study by Van den Bosch and colleagues performed in our hospital. No significant differences were observed between leucocyte differential cells counts in BAL fluid comparing results from 1983 and the current study [10]. Regarding the overlapping reference values presented by the BAL Cooperative Group Steering Committee [25], they were similar to cell counts described in our study. Of interest, none of the 55 subjects had plasma cells in their BAL fluid (Table 2), underscoring the diagnostic value of finding a plasma cell in BAL fluid [31]. Finally, BAL fluid cell counts were not substantially different between atopic and non-atopic healthy individuals.

Detailed analysis comparing cell counts of 'pooled' BAL fluid fractions (fractions I and II) with 'alveolar' fractions (fraction II) has not been published for disease, nor for healthy subjects. We demonstrated a different cell distribution in the bronchial and alveolar BAL fractions, indicating different airway compartments. These results are consistent with BAL fluid data in children described by Pohunek *et al.* [32]. Moreover, considering the significant differences we found for macrophage and lymphocyte cell counts between the pooled and alveolar samples, analysis of only the alveolar fraction instead of the pooled fractions is recommended in diagnostic work-up of ILD patients. In addition, this approach prevents contamination with commensal throat flora, epithelial cells and proteins such as lactoferrin and lysozyme from the bronchial fraction and may prevent alteration of cytokine and chemokine distribution from alveolar samples [29,33].

Although there is considerable information concerning systemic immune responses and how these change with aging, relatively little is known about compartmentalized immune surveillance in the lung. Here we show that the BAL fluid $CD4^+/CD8^+$ ratio correlated strongly with age. This is consistent with data described by Meyer *et al.* [34]. However, in contrast with their data, we found that the increase of the $CD4^+/CD8^+$ ratio in elderly subjects was due to a decrease of absolute $CD8^+$ cells instead of an increase of $CD4^+$ cells. The decrease of absolute $CD8^+$ numbers, in blood and BAL fluid, may represent the general decline of absolute T cell numbers with advancing age, in particular circulating naive $CD8^+$ T cells [35]. Absolute BAL fluid $CD4^+$ lymphocyte numbers, however, did not decrease with advancing age. In the lung this might be explained by relative accumulation of memory $CD4^+$ T cells due to cumulative antigenic stimulation at mucosal surfaces [36]. In agreement with the latter, a strong positive correlation was found between age and the proportion of BAL fluid $CD4^+$ lymphocytes that expressed the mucosal integrin CD103, which is believed to play a key role in retention of lymphocytes in mucosal epithelial tissues

Table 4. Expression of activation and regulatory surface markers on PB and bronchoalveolar lavage (BAL) fluid $CD4^+$ and $CD8^+$ lymphocytes from non-smoking healthy controls[†].

Markers	PB	BAL	*P*-value
$CD4^+$ lymphocytes			
CD25			
(%)	45·4 (32·3–73·1)	47·0 (34·0–60·1)	n.s.
(MFI)	66 (55–93)	63 (53–89)	n.s.
CD28			
(%)	99·9 (95·5–100)	94·1 (80·5–98·2)	0·0006
(MFI)	808 (621–989)	641 (121–871)	0·005
CD69			
(%)	2·9 (1·3–14·7)	69·8 (51·8–78·5)	<0·0001
(MFI)	30 (29–38)	79 (48–449)	<0·0001
VLA-1			
(%)	3·2 (0·7–15·1)	26·9 (15·4–53·6)	<0·0001
(MFI)	22 (21–22)	63 (45–131)	<0·0001
FoxP3			
(%)	7·2 (3·8–10·4)	9·4 (5·2–14·1)	0·007
(MFI)	32 (14–71)	122 (45–173)	<0·0001
$CD8^+$ lymphocytes			
CD25			
(%)	6·3 (4·0–23·6)	19·2 (9·7–27·1)	0·0014
(MFI)	35 (24–42)	31 (28–41)	n.s.
CD28			
(%)	85·3 (63·5–96·3)	65·1 (44·7–78·7)	0·0004
(MFI)	506 (378–746)	212 (72–354)	<0·0001
CD69			
(%)	3·3 (1·8–19·0)	84·4 (75·2–91·7)	<0·0001
(MFI)	39 (32–58)	69 (43–172)	0·0001
VLA-1			
(%)	3·8 (0·9–20·7)	80·1 (58·2–88·4)	<0·0001
(MFI)	20 (19–21)	189 (80–423)	<0·0001
FoxP3			
(%)	2·6 (1·4–6·1)	1·6 (0·6–5·6)	0·02
(MFI)	46 (36–60)	55 (36–97)	0·03

[†]$n = 14$, age = 30 (20–62). The expression of activation and regulatory surface markers was determined on lymphocytes from BAL fluid fraction II. Data are presented as median (10th–90th percentiles). FoxP3, forkhead box protein 3; MFI, median fluorescence intensity of the positive fraction; PB, peripheral blood; VLA, very late antigen-1.

[37,38]. This $CD103^+CD4^+/CD4^+$ ratio has been proposed as a potential novel diagnostic marker for sarcoidosis [12] and has been evaluated recently by our group [2].

Accumulation in the lung of lymphocytes with an activated phenotype has been described in T cell-mediated pulmonary inflammation [13,39,40]. Our data showed that T lymphocytes from BAL fluid of healthy controls expressed similar high levels of lymphocyte activation markers (CD69 and VLA-1), while these markers were not expressed on corresponding peripheral blood lymphocytes. Moreover, the expression of the nuclear transcription factor FoxP3, a master switch for regulatory T cells, was higher on BAL fluid lymphocytes than peripheral blood T cells. Our data support the concept of compartmentalization of immune cell populations in non-lymphoid peripheral tissue under non-inflammatory conditions. The $CD69^+VLA\text{-}1^+$ T cell

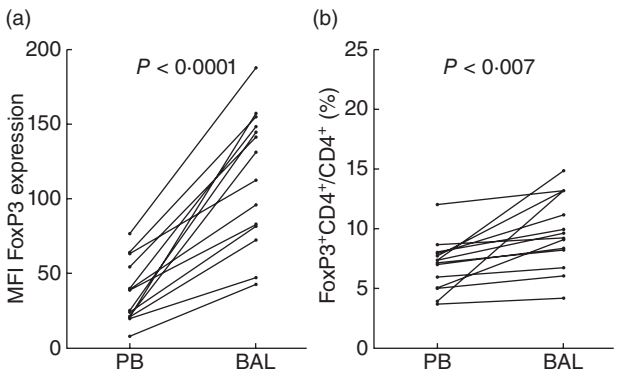

Fig. 4. Forkhead box protein 3 (FoxP3) median fluorescence intensity on $CD4^+$ cells [median fluorescence intensity of the positive fraction (MFI)] (a) and the proportion of $FoxP3^+$ positive $CD4^+$ cells (b) were significantly higher in BAL fluid than in peripheral blood samples.

phenotype and the increased expression of FoxP3 on BAL fluid T cells may be a consequence of the continuous exposure of the respiratory tract to environmental antigens. In contrast to FoxP3$^+$ cells, we showed recently that invariant NKT cell numbers were not compartmentalized in lungs from healthy adults [41], suggesting different roles of both immunoregulatory T cells in immune surveillance in peripheral tissue.

In summary, we recommend analysis of BAL fraction II in the diagnostic work-up of ILD patients. We established reference intervals of commonly and increasingly used cellular markers in this (alveolar) fraction of BAL fluid in a group of well-characterized healthy adults; age-related changes in differential cell counts, in particular T lymphocyte subsets, suggest that care must be taken to consider age when analysing BAL samples for clinical evaluation of interstitial lung diseases. The analytical validation of laboratory tests and pre-analytical standardization of BAL are prerequisites for further clinical validation and utility of BAL in interstitial lung diseases.

Disclosure

The authors have no conflicts of interest.

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